12.

Further Studies on the Susceptibility and Acquired Immunity of Marine Fishes to *Epibdella melleni,* a Monogenetic Trematode.

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(Plate I).

The presence of various degrees of susceptibility and resistance of marine fishes in the New York Aquarium to *Epibdella melleni* has been pointed out by Nigrelli and Breder (1934). This is the first instance of the development of an acquired immunity to an external trematode parasite.

The present preliminary paper deals with the actual count of parasites on the fish after successive exposures to infection and the determination of the degree of immunity developed.

The fishes employed for these experiments were two species of pompanos, *Trachinotus carolinus* and *T. falcatus.* The intensity with which these forms become infected is shown in Plate I. In this photograph of *T. falcatus,* small immature and larger matured parasites are easily noticed. The pompanos were collected at Sandy Hook Bay, N. J., brought into the Aquarium and kept in reserve tanks with running New York Bay water. As was pointed out by Nigrelli (1935 a), the trematodes are unable to live in sea water with a low specific gravity (i.e., 1.010-1.0128) like that of the Bay, so that the fishes at no time prior to the experiments were exposed to *Epibdella.*

After marking the animals, they were placed in the closed salt water system containing the parasites. In all the tests the counts were made at the end of 11-day periods. Before each count was made the fishes were removed from the tank, dipped for two minutes in a 10% solution of "sol-argentum" in fresh water and the parasites rubbed off. They were then allowed to rest for 3 days in tanks containing running Bay water. The results of these experiments are given in Tables I-V.

EXPERIMENTAL RESULTS.

In the first series, the common pompano, *T. carolinus,* was employed. The results have been previously reported as an abstract (Nigrelli, 1935 c).

Fish A (Table I) was given periodic injections of .2 cc. of serum taken from immunized pompanos of the same species. Although this fish outlived all the others in the series there are no definite indications that the resistance acquired was a passive one developed as a result of the injection of the serum. In this specimen complete immunity occurred after 5 successive exposures, or within 65 days. The resistance persisted for one year and two months, at which time the fish died of causes inherent in animals kept in captivity. The controls, Fish D and E, starting with a lighter initial infection, developed a resistance 14 days earlier and sustained it for 6 and 4 months respectively. One other control, Fish G, exposed to the infection 29 days after the experiment was started, obtained a light initial infection

TABLE I.

Number of worms present on *Trachinotus carolinus* at the end of each 11-day period.

	I	M	T	I	M	T*	I	M	T	I	M	T	I	M	T	I	M	T	L
A	606	250	856	270	0	270	143	26	169	16	13	39	15	0	15	0	0	0	1 yr., 2 mos.
B	423	279	702	290	0	290	296	58	354										50 days
C	117	59	176	85	0	85													21 days
D	169	76	245	60	0	60	69	45	114	33	5	38	0	0	0	0	0	0	6 mos.
E	112	122	234	75	0	75	44	14	58	12	16	38	0	0	0	0	0	0	4 mos.
F	164	134	298	130	0	130													21 days
G							287	101	388	35	13	48	0	0	0	0	0	0	8 mos.
H							25	3	28										12 days
I							30	5	35	12	3	15	0	3	3				45 days

* 5 day exposure; I, immature; M, mature; T, total; L, length of life of the fish after the experiment was started.

but developed a complete immunity at the end of second exposure and sustained it for 8 months. Fish B received a .5 cc. injection of ground dried worms suspended in salt water while Fish C received a similar amount of ground fresh worms. Here again the effects are not definite, in each case the fish dying early in the course of the experiment.

Although other species of *Epibdella* (e.g., *E. bumpusi* Linton on *Dasyatis centrura*) have been described from elasmobranchs, the present form has never been found on such hosts. It has long been known that very susceptible fishes kept in tanks containing dogfish, sharks, or rays either lack the parasites or become so lightly infected that no appreciable effects can be noted. Fish H and I in the above Table show this phenomenon, both of which were placed in a tank with many dogfish. Their early demise resulted from other unknown causes.

In the second series 3 fish (*T. falcatus*) were given intraperitoneally .1 cc., .25 cc. and .5 cc. respectively, of serum taken from an immunized grouper. In all cases a super-infection occurred as will be noted by the counts in Table II. The only reason for publishing this data is the interesting fact that from 6 fish (none over 4 inches in length) over *four thousand* worms were removed. Fish 3 of the controls, however, showed definite signs of developing a resistance, having an initial infection at the end of the first 11-day period of 774 worms, 384 in the second and 201 at the end of the third interval. Three days later after being re-exposed to infection the fish died. A count of the parasites yielded a total of 20 immature forms. It is altogether possible that some of the parasites had left the moribund or dead fish so that the last count may not be entirely accurate.

In the third series, one specimen of *T. falcatus* was injected with .25 cc. of serum taken from Fish G (*T. carolinus*) of the first experiment. The latter had sustained its resistance for 8 months, at which time it was used as a blood donor for the present experiment. The injected fish died at the end of the first period but the number of parasites was much lower than that found on the control. The latter lived for 4 periods and showed signs of acquiring a resistance to the parasites, although the count in the second interval was slightly higher than the first.

TABLE II.

Number of worms present on *Trachinotus falcatus* at the end of each 11-day period.

	I	M	T	I	M	T	I	M	T	I	M	T*	L
A′	502	165	667										14 days
B′	385	137	522										14 days
C′	702	151	853										14 days
1.	402	111	513										14 days
2.	582	170	752										14 days
3.	619	155	774	302	82	384	180	21	201	20	0	20	46 days

* Results of a 3-day infection.

The lower count resulting from the injection of immune serum might indicate that antibodies were formed which could be transferred to another fish to yield a passive immunity of a certain degree. The data obtained from the results of injection of sera from immunized fishes are not sufficient, however, to warrant the statement that definite protective antibodies are formed.

In the fourth series the two species of pompanos received periodically .25 cc. of either ground dried or fresh worms suspended in sea water. Like those recorded for Fish B and C in Table I, the results are unsatisfactory. However, Fish A″ (round pompano) did acquire a resistance at the end of the fourth exposure that lasted until the fish died 6 months later. This fish as well as Fish C″ (common pompano) was injected with .25 cc. of ground fresh worms before each exposure. The latter specimen, however, died before a complete immunity developed. Fish B″ and D″ (round and common pompanos respectively) died early in the progress of the experiment after receiving .25 cc. ground dried worms. The controls 1 and 2 also died before a complete immunity had developed, but definitely demonstrated the ability of these fish to build up a resistance to a super-infection. Superficially, the results of this experiment might indicate that the injection of ground fresh worms is effective in producing an immunity.

In another series of experiments (Nigrelli, 1935 b), an attempt was made to determine what part, if any, the mucus played in the protection of the fish to *Epibdella*. Accordingly, the parasites were removed, washed thoroughly in salt water and placed in petri dishes containing mucus from (1) immune grouper, (2) dogfish, (3) ray, (4) round pompano, and (5) salt water (control). The dogfish and ray show a natural immunity and never become infected, whereas the pompano is very susceptible. The parasites

TABLE III.

Number of worms present on *Trachinotus falcatus* at the end of each 11-day period. Fish T was injected with .25 cc. of serum taken from immunized Fish G (*T. carolinus*) shown in Table I.

	I	M	T	I	M	T	I	M	T	I	M	T	L
T	65	4	69										11 days
Control	233	76	309	255	60	315	181	13	194	22	2	24	59 days

TABLE IV.

Number of worms present on two species of pompanos at the end of each 11-day period. Fishes A″ and C″ were given .25 cc. of ground fresh worms. Fishes B″ and D″ were injected with a similar amount of ground dried worms. Fishes 1 and 2 are the controls.

	I	M	T	I	M	T	I	M	T	I	M	T	I	M	T	I	M	T	L
A″	122	37	159	83	21	101	20	6	26	3	8	11	0	0	0	0	0	0	6 Mos.
B″	172	60	232	110	41	151													25 days
C″	133	26	159	82	31	121	16	8	24	10	12	22	6	2	8	4	0	4	86 days
D″	162	76	238																
1.	192	123	315	132	82	214	104	30	134	62	15	77							58 days
2.	142	60	202	68	12	80	6	10	16										43 days

in the mucus from the dogfish were moribund in 3 hours, and in 5 hours practically all were dead.

Those in mucus from the immune grouper were moribund in 4 hours and dead in 8 hours. Most of the parasites in mucus from the ray were dead in 4 hours. Those in mucus from the pompano were kept alive from 18-24 hours, while those in sea water remained alive for 3 days.

Attempts were then made to see what effects the injection of such mucus in fish would have in the development of a resistance to the trematodes. The results are shown in Table V. Fish A_1″ (common pompano) and Fish B_1 (round pompano) each were given .5 cc. of mucus taken from skate and dogfish respectively. Both died 3 days following the injection. Fish C_1 and D_1 each received .5 cc. sea water suspension of mucus from ray and immune grouper. The former showed signs of developing a definite resistance, yielding a total of 597, 323 and 157 worms in each successive period. In the fourth trial, however, a definite breakdown of this partial immunity occurred for no apparent reason, producing 389 worms. This individual died two days later in Bay water. Fish D_1 also showed an almost complete immunity before it died 71 days later. Fishes 1 and 2 were used as controls. Here again, a definite resistance developed to successive exposures. Fish 2

TABLE V.

Number of worms found on two species of pompanos at the end of each 11-day period.

	I	M	T	I	M	T	I	M	T	I	M	T	I	M	T	I	M	T	I	M	T	L
A_1	167	0	167																			3 days
B_1	109	0	109																			3 days
C_1	482	115	597	273	50	323	136	21	157	302	87	389										58 days
D_1	503	112	615	180	70	250	101	14	115	20	1	21	10	14	24							71 days
1.	380	60	424	211	48	259	186	46	232	102	16	118										58 days
2.	413	72	485	170	33	203	80	10	24	0	6	6	2	3	5	0	0	0	0	0	0	125 days

shows this phenomenon very strikingly. The number of worms dropped from an initial count of 485 in the first period to 24 in the fourth. An extremely light infection persisted in the next 3 successive trials, finally yielding no worms in the last two trials (8 and 9). This fish died after 3 days in Bay water, in all probability from handling, to which this species is very sensitive.

Additional Observations on Susceptibility.

A large variety of fish hosts were found to be susceptible to *Epibdella melleni* in the New York Aquarium (Nigrelli and Breder, 1934). It was found at that time that all the hosts belong to the Order Acanthopteri or spiny-rayed fishes. Other forms belonging to this group that were not previously recorded and have since been found susceptible are *Therapon jarbua, Psettis argentus* and *Scatophagus argus,* all East Indian species. These forms become highly infected and are readily killed by the parasites. Another group found to be susceptible to a super-infection of the trematodes are the European wrasses. A collection of such fishes was wiped out within three weeks and a count from one fish (12 inches) at the end of one 11-day period yielded over 2,000 parasites in various stages of growth.

When the more susceptible fishes are removed from the closed circulation, those that formerly showed a natural immunity eventually take on a very light infection. Thus, in such instances various species of West Indian parrot fishes became infected. Three fish, examined at the end of one 11-day period, each produced 30, 21 and 80 small parasites. At such times, a few individual sea catfish (*Galeichthys milberti*) also became parasitized. In this form the infection is limited to the eyes (cornea) and never with more than a half-dozen worms. This is the only non-spiny rayed fish in the Aquarium to become infected with *Epibdella.*

Discussion.

The development of resistance to metazoan parasites has been demonstrated in several host species. Investigations of Ackert, Africa, Chandler, Cort, Fujinami, Miller, Ozawa, Stoll and others have shown definitely that an immunity is developed against such helminthic parasites as trematodes, cestodes and nematodes. Darling (1922) was the first to study this problem of metazoan immunity by making actual counts of hookworms after anthelminthic treatments. The disadvantages of this method for internal parasites are many. In 1923, Stoll developed a technique for counting the eggs of the parasites in faeces and showed that a correlation existed between the eggs per gram of faeces and number of worms parasitizing the host. This method (with slight modifications) is used today in studies on susceptibility and immunity of animals to internal metazoan parasites.

Miller (1931) was able to determine the degree of resistance that albino rats develop against infection with the larval tapeworm, *Cysticercus fasciolaris,* by counting the number and determining the size of the cysts formed in the liver.

In this series Miller has definitely shown that "An active acquired immunity to a metazoan parasite, *Cysticercus fasciolaris,* has been artificially produced in the albino rat by periodic injections of fresh or powdered worm material." Miller and Gardiner (1932) further demonstrated that a passive immunity occurred in rats by intraperitoneal injections of serum from infected rats or from those actively immunized against *Cysticercus fasciolaris.*

Nigrelli and Breder (1934) have shown that a large number of fishes become infected with *Epibdella.* In analyzing this data they found that (a) some fish acquire a total immunity lasting for long periods; (b) some acquire a partial immunity; (c) certain fishes that appear to have a natural immunity will become slightly infected during periods of epidemics; and (d) certain fishes are always susceptible and may show a heavy or light infection. In one form, the black angelfish, an apparent inverse age immunity appeared to be present. In this case the young acquired an immunity after a very short period of susceptibility (one to two weeks) while the older members were always parasitized. It has been further shown that in the moonfish, *Vomer setapinnis*, a skin immunity is present. Re-infection never takes place in the region of the previous infection, indicating a tendency toward localized immunity.

The existence of definite immunity to *Epibdella melleni* is further demonstrated in the present studies by actual count of the parasites after successive exposures to re-infection. The attempts to produce a resistance by periodic injections of ground dried and fresh worms gave indefinite results. In each case the controls were equally or more effective in producing a partial or complete immunity after several exposures. The injections of sera from immunized fishes were equally ineffective. The effects, if any, resulted in a breakdown of any inherent resistance so that the initial infection was in the majority of the cases higher than that of the controls. This is shown in Tables I (Fish A) and II. The individual shown in Table III, however, developed a very light initial infection, with only 69 worms present at the end of one 11-day period. This fish was injected with .25 cc. of serum taken from Fish G (Table I) which had developed a complete resistance at the end of the second exposure and had sustained it for 8 months, or up to the time it was used as a blood donor. As pointed out by Taliaferro (1934), although no protective antibodies can be demonstrated in many of the acquired helminthic immunities, "Extreme caution must, however, be exercised in excluding antibodies because most of the immunities are probably local and may involve antibodies produced locally which when diluted in the blood stream are not demonstrable." The parasiticidal action of fish mucus is very indicative of this local phenomenon. It may be that immune bodies are produced locally and sent out with mucus secretion, or as in some instances (elasmobranchs) the mucus itself may be toxic to the parasites. The fact that in the sea catfish the parasites are limited to the eyes is very suggestive. It is well known that there are no mucoid secreting cells around the eyes of fishes. Further experiments along these lines are now in progress.

Summary.

1. Two species of pompanos (*Trachinotus carolinus* and *T. falcatus*) are shown to be very susceptible to infection with *Epibdella melleni*, a monogenetic trematode of marine fishes.

2. These fishes may acquire a permanent or partial resistance to the parasites after several exposures to the infection.

3. The results obtained from injecting dried and fresh worm material, as well as sera from immunized fishes, are unsatisfactory. In each series attempted, the controls developed either a partial or complete immunity which was just as effective, if not more so, than those subjected to the injections.

4. It has been found that worms placed in petri dishes containing mucus taken from elasmobranchs and immunized fish will die in a shorter time than when placed in mucus taken from a susceptible fish or when kept in sea water. Such mucus, however, when injected into susceptible fish gave indifferent results.

5. Other spiny-rayed fishes, not previously recorded, were found to be susceptible to the infection. The sea catfish (*Galeichthys milberti*) is the first and only non-spiny rayed fish to become infected. However, the infection is very light and always limited to the eyes.

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EXPLANATION OF THE PLATE.

Plate I.

Fig. 1. *Trachinotus falcatus* (Round pompano), showing a super-infection with *Epibdella melleni*, an ectoparasitic trematode. 2x natural size.